Genotypic and phenotypic characterization of antimicrobial resistance patterns of salmonella strains isolated from raw milk in Sebeta, Ethiopia

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Abstract
Salmonella was isolated from 16% of the sampled cows. Generally, all the isolates showed resistance to the tested 9 antimicrobials drugs. The most common resistance was to Sulphamethoxazole (87.5%), Erythromycin (87.5%), Streptomycin (37.5%), Spectinomycin (56.3%), Tetracycline (31.25%) and Ampicillin (25%). Most of the isolates were relatively sensitive to Ciprofloxacin, Ceftriaxone, and Chloramphenicol. Among the 16 isolates, 56.25% of them show multi drug resistance to more than two antimicrobials. Multi drug resistant phenotypes identified were ACEShSuT, AESuT, AESuT, CEShSu, ESShSu, SSHuT, AESu, ESSu and ShSuT. Among sulphamethoxazole resistant isolates, 40% of isolates carried sul1 gene. In tetracycline resistance isolates, tetA was found in the highest frequency (60%) followed by tetC (20%). Approximately 83% of the streptomycin resistant isolates harbored strA gene.

Keywords: Salmonella, salmonellosis, zoonoses, antimicrobial resistance and gene.

Introduction
Salmonellosis is one of the most widespread food-borne zoonoses in Ethiopia (Molla et al., 1999). Dairy cows are one of and major reservoir for Non Typhoidal Salmonella in industrialized countries and large outbreaks of Salmonella infection have been associated with food-borne transmission including that from contaminated poultry and poultry products, meat, and milk and other dairy products (Threlfall, 2000).

The use of antimicrobial drugs in animal production and drug prescription haphazardly in veterinary as well as in human medicine are the two major predisposing factors for emergence of resistant Salmonella (Acha and Szyfres, 2001). Development of antimicrobial resistant Salmonella has economical as well as public health importance this is due to raise health care costs as a result of treatment failure (Gorbach, 2001) and constraints of therapeutic drug selection obtainable to veterinarians and physicians for therapy of salmonellosis (Witte, 1998).

Research performed in Ethiopia revealed that significant prevalence of Salmonella both in human as well as in animals however reports from seemingly healthy lactating dairy cows and characterization of the responsible resistant target gene is very limited and almost negligible in the latter case. With regards to the concern of antibiotic resistance, due to the lack of information on the trend of the prevalence of Salmonella and the associated drug resistance in developing countries like Ethiopia, it is difficult to talk about it. Besides the mentioned fact, identification and characterization of target resistant gene was not done.
previously. Thus the present study was designed and executed with the aim to determine the prevalence and antimicrobial sensitivity pattern of *Salmonella* from seemingly healthy lactating cows and to identify antibiotic resistant target gene by cultural, biochemical and molecular techniques.

**Material and Methods**

**Sample collection, Isolation and identification**

A total of 100 apparently healthy lactating exotic cows were randomly included in the study from 40 different households found in Sebeta, Ethiopia. About 15 -20 ml milk was aseptically collected at midstream from each animal using screw capped universal bottles and transported using Ice box to the National Animal Health Diagnostic and Investigation Center (NAHDIC) laboratory for isolation and identification tests. The milk samples were tested for the presence of *Salmonella* spp. using selective enrichment and isolation protocol recommended by International Organizations for Standardization (ISO – 6579 - 2002).

**Biochemical confirmation**

*Salmonella* isolates were preliminary identified by biochemical tests viz. Indole, Methyl red, Voges proskauer and L-lysine decarboxylation. The isolates were further characterized for their biochemical activity by the following tests viz. urea hydrolysis and production of H$_2$S on TSI (WHO, 2003).

**Antimicrobial Susceptibility testing**

The antimicrobial susceptibility test was performed in the National Veterinary Institute (NVI), Debre Zeit Ethiopia. The bacterial isolates were subjected to *in vitro* antibiotic sensitivity test as per the method of WHO (2010). The antibiotic discs were obtained from Clinical Diagnostic Ltd. Bangkok. Isolates were tested against commonly used antibiotics in human medicine as well as veterinary set up viz. Streptomycin (S10µg), Ceftriaxone (CRO30µg), Erythromycin (E15µg), Ciprofloxacin (CIP5µg), Tetracycline (TE30µg), Sulphamcthoxazole (RL25µg), Chloramphenicol (C30µg), Spectinomycin (SH10µg), Ampicillin (AMP10µg). Isolates were inoculated to Nutrient broth overnight and plates of Mueller Hinton (MH) agar were seeded with about one ml of inoculum. The inoculum was allowed to dry. Antibiotic discs were placed on the inoculated agar surface at about two cm apart. The plates were incubated at 37°C overnight and diameter of the zones of inhibition was measured. The measurements were compared with zone size interpretative chart furnished by Clinical Laboratory and Standard Institute (CLSI) Guideline M100-S17 and the zones were graded as sensitive and resistant.

**DNA Extraction and PCR condition**

The *Salmonella* DNA templates were extracted according to Wilson (1987) with minor modifications. Molecular characterization of target antimicrobial resistant genes, which were identified phenotypically, was done by using standard PCR. Primer sequences and predicted sizes for PCR amplicons of different antimicrobial resistance genes from *Salmonella* are in Table - 1. The PCR amplicons were sequenced and the resulting nucleotide sequences were blasted in the NCBI database using blastn program. Sequence matches shared 96–100% homology with the corresponding antimicrobial resistance nucleotide sequences. The antimicrobial resistance genes are genes encoding for the tetracycline efflux proteins (*tetA* and *tetC*); streptomycin phosphotransferases (*strA*) and dihydropteroate synthetase (*sul1)*.

PCR was carried out in a final reaction volume of 15 µl using PCR tube. A master mix (4mM MgCl$_2$, 0.4mm of each dNTPs (dATP, dCTP, dGTP, dTTP), 0.05units/ml of Taq DNA polymerase, 150 mm tris-HCl PCR buffer) for minimum of 6 samples was prepared and aliquoted in 14 µl quantities in each PCR tube. One µl sample of DNA was added in each tube to
make the final volume of 15 µl. PCR tubes containing the mixture were tapped gently and quickly spun at 10,000 rpm for a few seconds. The PCR tubes with all the components were transferred to thermal cycler (Eppendorf, Germany).

PCR conditions used for the amplification of DNA as described by Wain et al., (2003): Initial denaturation at 95°C for 5 min followed by 28 cycles of 95°C for 30s, 57.5°C for 1 min, and 72°C for 2 min.

The PCR products were analyzed by electrophoresis with 1.5% and/or 0.7% agarose (Bio-Rad) in 1×Tris–boric acid–EDTA buffer. 1µg/mL ethidium bromide and visualized bands were photographed using a Kodak scientific imaging system version 3.6.1 (Kodak, USA). A molecular weight standard ladder was included on each gel.

Results

Bacteria Isolation and Antimicrobial Susceptibility profile

Salmonella isolates were detected in 16 % of the total milk sample tested. The number and percent of Salmonella isolates resistant to different antibiotics are observed in Table - 2. All the sixteen isolates of Salmonella, from dairy cows were exposed to a group of nine antibiotics. Generally all the isolates showed resistance to the tested 9 antimicrobials drugs.

Ciprofloxacin is effective against all the isolates (100%), ceftriaxone (93.8%), and chloramphenicol (87.5%). A high proportion of the isolates showed moderately sensitive to ampicillin (43.8%), spectinomycin (37.5%), streptomycin (50%) and tetracycline (31.3%). However, almost all Salmonella showed least sensitive to sulphamethoxazole (6.3%) and none of them are sensitive to erytromycin as indicated in Table - 2.

The most common resistance was to sulphamethoxazole (93.73%), erythromycin (87.5%), streptomycin (37.5%), spectinomycin (56.3%), tetracycline (31.25%) isolates were resistant for at least two antimicrobials, 9 (56.25%) of them shows multi drug resistance to more

<table>
<thead>
<tr>
<th>Target Gene/Primer</th>
<th>Oligonucleotide sequence (5’→3’)</th>
<th>Fragment size (bp)</th>
<th>Annealing Temp. (°C)</th>
<th>Reference sequence (Gene bank Accession number(s))</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetA</td>
<td>f:-ATGAATAGTTCGACAAAGATCG r:-CTAAGCACTTTGCTCTCCTTAC</td>
<td>1206</td>
<td>57.1</td>
<td>Salmonella Typhimurium plasmid R64</td>
</tr>
<tr>
<td>tetC</td>
<td>f:-ATGGAAAAATAAAAATCATCAACA r:-TTATTATCTTCTCAAGCTCAAT</td>
<td>594</td>
<td>52.1</td>
<td>TC71 tetC gene AB089595.1</td>
</tr>
<tr>
<td>strA</td>
<td>f:-ATGCCCTCAGGCAAAT r:-TCAACCCCAAGTCAGAGG</td>
<td>704</td>
<td>51.6</td>
<td>Salmonella Typhimurium plasmid pSLT-BT</td>
</tr>
<tr>
<td>sul1</td>
<td>f:-ATGGTGGCGGTGTTCG r:-CTAGGCATGATCTAACCCTC</td>
<td>840</td>
<td>56.7</td>
<td>Salmonella Typhimurium EF592571.1</td>
</tr>
</tbody>
</table>
than two antimicrobials as it shown in the table 3 below. *Salmonella* isolates that showed resistance to three antimicrobials presented below.

Six different resistance patterns were observed (Table - 3). One heptaresistant and one pentaresistant isolate with ACESShSuT and AESuT pattern, respectively. Four (25%) tetraresistant isolates with four different resistance pattern; AESuT, CESShSu, ESShSu and ShSuT. A total of 3 (18.75%) isolates were resistant to three antimicrobials with three different resistance pattern; AESu, ESSu and ShSuT. Five (31.25%) *Salmonella* isolates were resistant to two antimicrobials with two different resistance patterns (four isolates with ESu and one isolate with ES).

### Table – 2. Number of isolates which are sensitive, intermediate and resistant; and their relative percentage

<table>
<thead>
<tr>
<th>Antimicrobials Tested</th>
<th>Number of isolate (%)</th>
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<tbody>
<tr>
<td></td>
<td>Sensitive</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>16 (100)</td>
</tr>
<tr>
<td>Ampicilline</td>
<td>7 (43.8)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>15 (93.8)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>14 (87.5)</td>
</tr>
<tr>
<td>Sulphamethoxazole</td>
<td>1 (6.25)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>5 (31.25)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>6 (37.5)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>8 (50)</td>
</tr>
</tbody>
</table>

### Table – 3. Percentage of multiple drug resistant bacteria

<table>
<thead>
<tr>
<th>Number of antimicrobial resistance</th>
<th>Antimicrobial resistance pattern (number of isolates)</th>
<th>Number of isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>one</td>
<td>Sh (1) Su (1)</td>
<td>2(12.50)</td>
</tr>
<tr>
<td>two</td>
<td>ES (1) ESu (4)</td>
<td>5(31.25)</td>
</tr>
<tr>
<td>three</td>
<td>AESu (1) ESSu (1) ShSuT (1)</td>
<td>3(18.75)</td>
</tr>
<tr>
<td>four</td>
<td>AESuT (1) CESShSu (1) ESShSu (1) SShSuT (1)</td>
<td>4(25.00)</td>
</tr>
<tr>
<td>five</td>
<td>AESuT (1)</td>
<td>1(6.25)</td>
</tr>
<tr>
<td>seven</td>
<td>ACEShSuT (1)</td>
<td>1(6.25)</td>
</tr>
</tbody>
</table>

**Key to Abbreviations:** A (ampicillin), S (streptomycin), T (tetracycline), C (chloramphenicol), E (Erythromycin), Sh (Spectinomycin), Su (Sulphamethoxazole)
Characterization of resistance gene

All Salmonella isolates carried one or more antimicrobial resistance genes in different combinations. The majority (60%) of multidrug resistant Salmonella carried antimicrobial resistance gene sequence. In total, five Salmonella isolates carried one antimicrobial resistance genes, one isolate carried two resistance genes and three isolates harbored three antimicrobial resistance genes in different combinations. Antimicrobial resistance genes sul1 and strA were detected in the highest frequency in 40% and 33.3% of the total isolates, respectively. The tetA and tetC gene were identified in 20% and 6.67% of the isolates, respectively (Table - 4).

Discussion

In the present finding the prevalence of Salmonella is higher (16%) than a report from study by Mekonen et al. (2011) on seemingly healthy dairy cows and other reports from meat. Thus, milk collected from these lactating cows could predispose the household and the community at large for Salmonella infection. This higher prevalence from milk in this study is an alarm to the dairy company and could predispose the community to food poisoning outbreaks in large scale.

A report by Mekonnen et al. (2011) with a prevalence of 10.76 % from seemingly healthy dairy cows is less than the current funding. The discrepancy may be due to the variation in the experiments employed and also the trend of increasing in prevalence through time due to lack of appropriate prevention and control measures. A similar study by Alemayehu et al. (2003) also revealed a prevalence of 7.1% of slaughtered cattle from abattoirs.

All the Salmonella isolates showed at least antimicrobial resistance to a single antibiotic. This finding clearly indicated that due to the treatment failure as a result of drug resistance, food poisoning outbreaks would be difficult to treat this represents public health hazards. Multiple drug resistance (81.75 %)
was investigated in the present study was almost the same with finding of Mekonnen et al. (2011) (83.3%). The present finding is higher than other studies conducted in Ethiopia except Mekonnen et al. (2011) and elsewhere in the world. The reason for this discrepancy may be as a result of the escalating rate of improper use of antimicrobials in the livestock production.

Mekonnen et al. (2011) and Endrias et al. (2009) reported that the most Salmonella isolates from livestock products and individual who consumed contaminated food from Addis Ababa were resistant to antimicrobials. They also reported that these isolates develop resistance to commonly used antimicrobials in veterinary as well as public health including streptomycin, ampicillin, and tetracycline. The outcome of the present experiment also revealed resistance and intermediate resistance of Salmonella isolates to routinely employ antibiotics including sulphamethoxazole, erythromycin, streptomycin, spectinomycin, tetracycline and ampicillin. This implies the isolated organisms are not inhibited by the usual achievable concentration of the drugs with normal dosage schedule.

Out of the 16 resistant isolates 14 (87.5%), 6 (37.5%), 5 (31.25%) and 4 (25%) showed resistance for sulphamethoxazole, streptomycin, tetracycline and ampicillin, respectively. The notably rise in occurrence of antimicrobial resistance in Salmonella for the mentioned antibiotics was most likely a sign of their common use both in veterinary as well as human medicine. Owing to the relatively limited access and high price to get the newly developed cephalosporins and quinolone drugs, the boom prevalence of antimicrobial resistance Salmonella to a relatively low-priced and regularly available antibiotics are alarming (D’Aoust, 1989) for low income society living in most developing countries like Ethiopia. Another important aspect is the spread of multidrug-resistant (MDR) S. typhimurium DT104. (Threlfall, 2000). Multi drug resistant phenotypes identified were ACESShSuT, AESSuT, AESuT, CEShSu, ESShSu, SSSuT, AESu, ESSu and ShSuT.

The present finding on resistance showed that resistance to various broad spectrum Cephalosporins (Ceftriaxone), Aminoglycosides, newer Quinolones (Ciprofloxacain) and Chloramphenicol was absent with the exception of the 6.3% intermediate resistance to Ceftriaxone, possibly owing to their restricted application in Ethiopia for therapeutic purpose both animals and human beings. A report from central part of Ethiopia among isolates of sheep and goat by Molla et al. (2006) also reveal the same finding, and Mekonnen et al. (2011) also reported from the dairy farms of Addis Ababa.

Analysis of PCR amplified fragments of antimicrobial resistance genes from Salmonella isolates evaluated in this study revealed that the majority of Salmonella isolates carried multiple resistance genes in different combinations. The multi drug resistance is due to the isolates harbored tetA, tetC, strA and sul1 genes encoding Tetracycline, Streptomycin and Sulphamethoxazole resistance, respectively.

Widespread resistance to sulphamethoxazole was observed in isolates possessing sul1 gene. Among sulphamethoxazole resistant isolates, 40% of isolates carried sul1 gene. And hence, in this observation sul1 gene in Salmonella isolates was the resistance mechanism to sulphamethoxazole. A similar observation was made in Salmonella enterica strains in Portugal (Patricia et al., 2005).

One of the common resistances observed in Salmonella isolate was to tetracycline. Tetracycline resistance was mediated by the tet gene which is efflux gene. Tet genes express membrane-associated proteins which reduce the intracellular drug concentration by exporting tetracycline out of a cell and thereby protect the ribosomes within the cell. The majority of the
resistant isolates in the present experiment carried tetA. Among tetracycline resistance isolates, tetA was found in the highest frequency (60%) followed by tetC (20%). The tetA gene is located frequently on transposons such as Tn1721, and the gene has been found to be widespread among Gram negative bacteria including \textit{Salmonella} (Pasquali \textit{et al.}, 2005).

Among streptomycin resistant isolates, 83.33% of the isolates harbored strA. Similarly, Kikuvi \textit{et al.} (2010) reported strA were detected in streptomycin resistant isolates from pigs in Kenya Slaughter house. The gene strA, which may be part of transposon Tn5393, has been found frequently among streptomycin-resistant isolates, such as \textit{Salmonella} Typhimurium (Popoff \textit{et al.}, 2003).

In conclusion, the current finding reveals that higher proportion of \textit{Salmonella} isolates developed resistance to the commonly prescribed antimicrobials for human being as well as animals and this may be a considerable risk in the treatment of clinical cases. Thus, further molecular epidemiology has to be planned in order to know the source of resistance and the possible means of resistant gene transfer within and across species of bacteria. Moreover, prudent control measures are instituted to combat the ever increasing situation of antimicrobial resistance.

\textbf{References}


